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Mucor irregularis, a first record for South America

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Abstract—During a survey on the diversity of *Mucorales* in areas of Atlantic Forest and Caatinga (semiarid) in the state of Pernambuco, Brazil, two specimens of *Mucor irregularis* were isolated from soil and the roots of *Sorghum bicolor*. Their identity was confirmed by morphophysiology and ITS rDNA sequence analysis. The specimens are described and illustrated. This is the first record of *M. irregularis* in South America.

Key words—endophytic fungi; phylogenetic analysis, *Mucoromycotina*, taxonomy

Introduction

Mucor was erected by Fresenius in 1850 to include species that produce simple or branched sporangiophores that arise directly from the substrate, as well as globose or subglobose non-apophysate sporangia (Benny 2014). *Mucor* species are distributed around the world and are commonly found in soil and organic substrates, including stored grains, vegetables, and herbivore dung (Santiago & al. 2013, Lima & al. 2016). Several species of biotechnological importance are used in industrial processes, including the production of fermented foods (Abe & al. 2004, Millati & al. 2005). In contrast, *Mucor* taxa reported to cause human infection are regarded as among the most important clinical pathogens in *Mucorales* (Hoog & al. 2000, Ribes & al. 2000, Álvarez & al. 2009).

Mucor irregularis has been described as an emerging opportunistic fungus, especially in Asian countries. Its cutaneous or subcutaneous clinical infection affects faces and exposed extremities of immunocompromised patients, becoming chronic and leading to severe mutilation or patient death (Li & Lun 2012, Lu & al. 2013). Cases were initially reported in China, where *M. irregularis* (as *Rhizomucor variabilis* R.Y. Zheng & G.Q. Chen) was first isolated and described from a patient's lesions (Zheng & Chen 1991). Subsequent cases were reported from Australia (Ribeiro & al. 2010), Japan (Tomita & al. 2011), India (Hemashettar & al. 2011) and the USA (Schell & al. 2011). *Mucor irregularis* has also been isolated both from the soil and as an endophyte of *Rhizophora stylosa* Griff. (Lu & al. 2013, Gao & al. 2016). Nguyen & al. (2016) reported the isolation of *M. irregularis* from the gut of soldier fly larvae in Korea.

Mucor irregularis has been included in phylogenetic analyses by Álvarez & al. (2011), Walther & al. (2013), and Lu & al. (2013). Lu & al. (2013), who provided a detailed taxonomic and phylogenetic study based on four gene loci of *M. irregularis*. Focusing on geographic distribution, they reported that while *M. irregularis* is predominantly found in East Asia, it is distributed around the world. Its presence remains unconfirmed in Europe and South America, however.

The purpose of this manuscript is to describe and illustrate two isolates of *M. irregularis*, one from soil samples collected in the Atlantic Forest and the other as an endophyte from the roots of *Sorghum bicolor* (L.) Moench in crop systems in the semiarid region of Brazil.

Materials & methods

Soil and root collection

Soil samples were collected in the Saltinho Biological Reserve (8°43′09″S 35°10′11″W), located 100 km from Recife in the municipality of Tamandaré, Pernambuco, Brazil, which contains an area of Atlantic Forest. The climate is characterized as humid (As'; Köppen 1936) with a mean annual temperature of 25°C (Andrade & Moura 2011). Healthy roots of *Sorghum bicolor* were collected in crop areas of the Instituto Agronômico de Pernambuco (the Pernambuco Agronomic Institute; IPA) in Serra Talhada, Pernambuco, Brazil (7°59′00″S 38°19′16″W). The climate of this region is characterized as semiarid (BSwh'; Köppen 1936) with a mean annual temperature of 25°C (Almeida & al. 2011).

Isolation of *M. irregularis* from the soil and from healthy roots of *Sorghum bicolor*

Five milligrams of soil were added to Petri dishes containing a wheat germ agar (WGA; Benny 2008) augmented with chloramphenicol (100 $mg.L^{-1}$). Root fragments

were cut and disinfected with 70% alcohol for 1 min, 2% sodium hypochlorite solution (NaOCl) for 2.5 min, and 70% alcohol for 30 s; after being washed twice with sterilized distilled water (Araújo & al. 2002), the root fragments were transferred in triplicate to Petri dishes containing malt extract agar (MEA; Benny 2008) plus chloramphenicol (50 mg.L^{-1}) to prevent bacterial growth.

The growth of colonies from the soil and roots was monitored for 96 hours at room temperature (26 ± 2°C) and subjected to alternate light and dark periods. In order to purify the *M. irregularis*, fragments of the mycelium were transferred separately to the malt extract agar medium (Benny 2008) augmented with chloramphenicol (80 mg.L^{-1}).

Morphological and physiological identification and mating experiments

Pure cultures from the isolates were cultured in triplicate on MEA and potato dextrose agar (PDA) and incubated at 15, 20, 25, 30, and 35°C for 15 days. Fragments of mycelia were removed from cultures, placed on microscope slides with KOH (3%), and observed under a Zeiss Axioscope 40 light microscope. The specimens were identified by observing their macroscopic (color, appearance, and diameter of colonies) and microscopic characteristics as described by Zheng & Chen (1991) and Lu & al. (2013). Colors of colonies were designated according to Maerz & Paul (1950). Mating experiments with the URM 7723 and URM 7724 strains were carried out on three MEA plates at 25°C. A 5-mm diameter disk was cut from each strain and placed on opposite sides of the plates, 3 cm apart. After three weeks the plates were checked for zygospores using a Leica EZ4 stereomicroscope with 95% ethanol added to each culture to facilitate observation.

Extraction and purification of rDNA

Fungal biomass was obtained from MEA cultures in test tubes stored at 28°C for up to 6 days and transferred to 2 mL microtubes with screw caps with 0.5 g of acid-washed glass beads with two different diameters (150–212 µm and 425–600 µm, 1:1; Sigma, USA). The material was crushed by stirring at high speed in a FastPrep homogenizer. Genomic DNA was extracted as described by Góes-Neto & al. (2005). The mycelium was washed with chloroform: isoamyl alcohol (24:1) and then homogenized in CTAB lysis buffer (2% cetyltrimethylammonium bromide, 20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl; Doyle & Doyle 1987, 1990). The DNA was precipitated in isopropanol, washed with 70% ethanol, and resuspended in 50 µL ultrapure water.

The primer pairs ITS1/ITS4 (White & al. 1990) were used to amplify the rDNA ITS1–5.8S–ITS2 region. The polymerase chain reaction was conducted as described by Oliveira & al. (2014). The final amplicons were purified with the Invitrogen™ PureLink™ PCR Purification Kit, sequenced directly or cloned with a CloneJet™ PCR Cloning Kit, in accordance with the manufacturer's instructions, and sequenced. The newly obtained sequences were deposited in GenBank.

Phylogenetic reconstructions were obtained by analyzing the ITS1–5.8S–ITS2 sequence data. The fungal sequences were aligned with Clustal X (Larkin & al. 2007)

and edited with BioEdit (Hall 1999). Prior to phylogenetic analysis, the optimal nucleotide substitution model was estimated using Topali 2.5. Bayesian inference analysis (two runs over 1×10^6 generations with a burn-in of 2500) was performed using MrBayes 3.1.2, and maximum likelihood analysis (with support estimated by bootstrap analysis with 1000 replicates) was performed using PhyML (Guindon & Gascuel 2003), launched from Topali 2.5.

Phylogenetic results

In BLASTn analysis, our new Brazilian sequences MG269827 and MG269828 had a 99% identity with *Mucor irregularis* JX976247 (CBS 700.71) and a 96% identity with *M. irregularis* JN206150 (CBS 103.93, the ex-type strain of *Rhizomucor variabilis*). Phylogenetic analysis of the sequence datasets (Fig. 1) showed that our fungal sequences MG269827 (URM 7723) and MG269828 (URM 7724) were grouped with the sequences of *M. irregularis*.

Taxonomy

Mucor irregularis Stchigel, Cano, Guarro & E. Álvarez, Medical Mycol. 49: 71. 2011. Fig. 2

≡ *Rhizomucor variabilis* R.Y. Zheng & G.Q. Chen, Mycosystema 4: 47. 1991; non *Mucor variabilis* A.K. Sarbhoy 1965.

Colony initially white, becoming yellowish to cream (MP 19D1) with yellowish reverse (MP 11J6), reaching 9.4 cm in diam. and 9 mm in height after 4 days in MEA at 25°C. Sporangiophores erect, sympodially and monopodially branched, arising from aerial hyphae (3–)5–15.5(–26.5) µm diam., hyaline. Sporangia globose to subglobose, (16–)28–40(–50) µm diam., grayish brown, with diffluent wall. Columellae globose 12.5–26.5 µm or ellipsoidal to cylindrical 30–33.5 × 22–30 µm, rarely conical 25–40 × 20–35 µm, some variable in shape, brownish, smooth-walled; collar small, little evident. Sporangiospores hyaline, smooth-walled, mostly ellipsoidal 2.5–11 × 2–4.8(–10), subspherical to ovoid 3.7–9.5 × 2.5–7 µm, or globose, 2.5–9.5 µm diam., some irregularly shaped and to 12 µm diam. Rhizoids observed. Chlamydospores and oidia observed. Zygosporangia not observed.

Specimens examined: **BRAZIL**, **Pernambuco**, **Tamandaré**, Saltinho Biological Reserve, 8°43′09″S 35°10′11″W, soil, 2016, D.X. Lima. (URM 7723; GenBank MG269827); **Serra Talhada**, IPA, 7°59′00″S 38°19′16″W, a healthy *Sorghum bicolor* root, 2016, R.J.V de Oliveira (URM 7724; GenBank MG269828).

Habitat & distribution: Recorded from soil in an Atlantic Forest area in Pernambuco, northeastern Brazil and as endophytic in healthy roots of *Sorghum bicolor* in the crop systems in the semiarid region of Pernambuco.

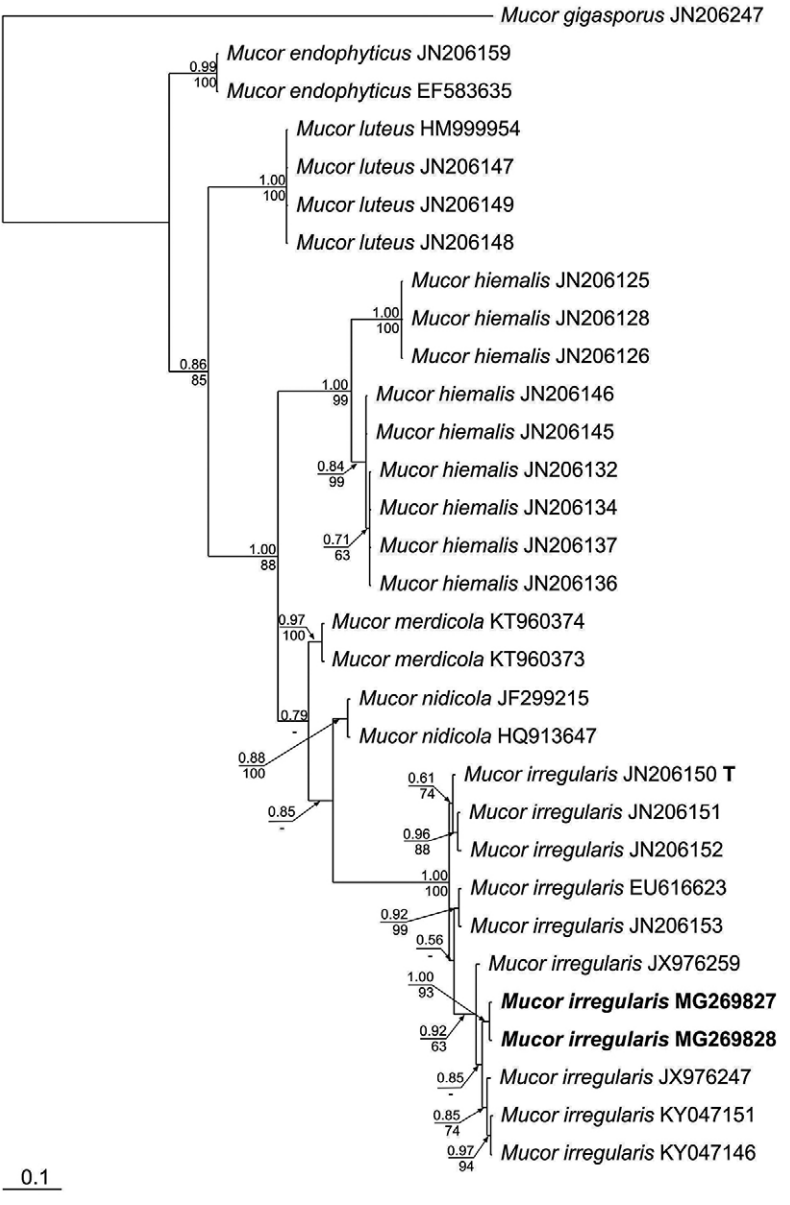

Fig. 1. Phylogenetic tree of *Mucor irregularis* and related species constructed using sequences of the ITS region. *Mucor gigasporus* was used as outgroup. Sequences are labeled with their database accession numbers. Support values are from Bayesian inference and maximum likelihood analysis. Our new Brazilian sequences are in boldface. Ex-type sequence of *M. irregularis* is annotated with T.

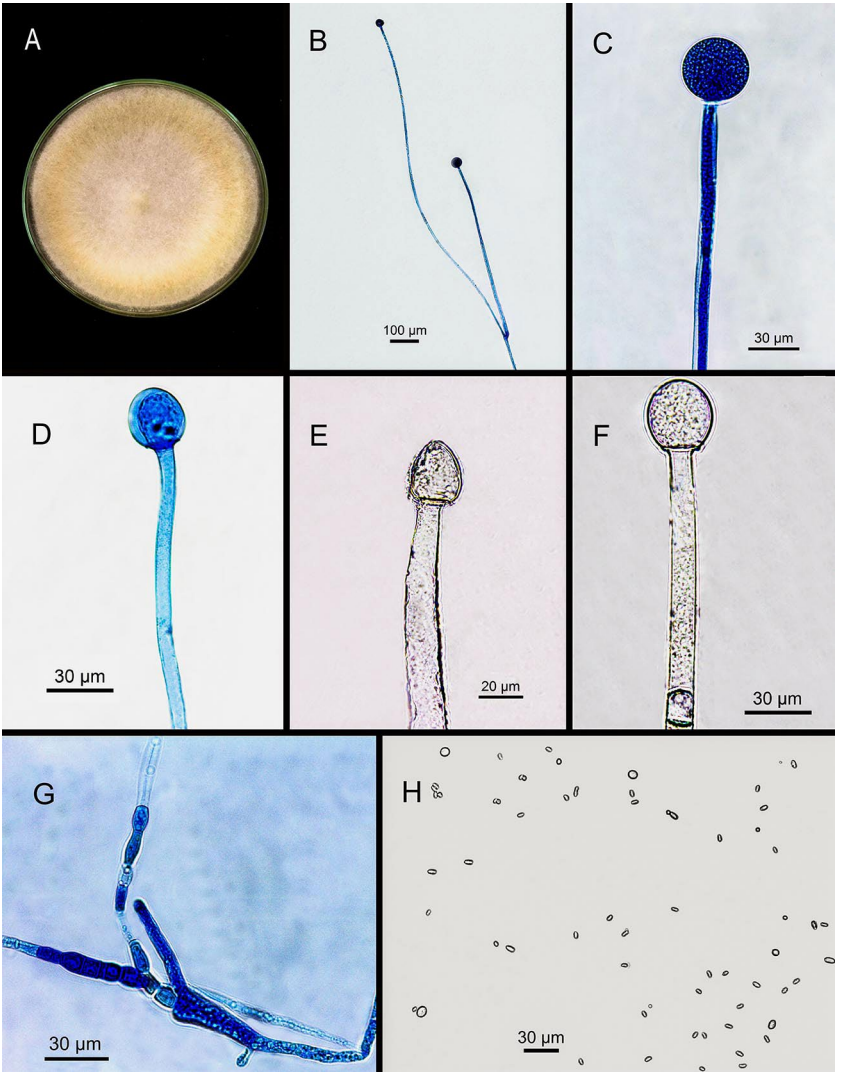

Fig. 2. *Mucor irregularis* (URM 7723): A. Colony; B. Sporangiophore with simple branch; C. Sporangiophore simple with sporangia; D. Sporangiophore simple with globose columella; E. Sporangiophore simple with conical columella; F. Sporangiophore simple with ellipsoidal to cylindrical columella; G. Chlamydospores; H. Sporangiospores.

Previously reported from Australia (Ribeiro & al. 2010), China (Zheng & Chen 1991, Lu & al. 2009, Zhao & al. 2009, Li & Lun 2012), Japan (Tomita

& al. 2011), India (Hemashettar & al. 2011), Nigeria (Lu & al. 2013), South Korea (Nguyen & al. 2016), USA (Schell & al. 2011). This is the first report from South America.

Discussion

The morphological characteristics of *M. irregularis* described here show close similarity to the original description given by Zheng & Chen (1991, as *Rhizomucor variabilis*). However, we did not observe in our material the variably and irregularly shaped columellae cited in the original description; both Brazilian isolates exhibited globose and ellipsoidal to cylindrical columellae and mostly ellipsoidal sporangiospores, some of which were cylindrical or globose as reported by Lu & al. (2009, 2013) and Schell & al. (2011).

In the ITS rDNA phylogenetic tree (Fig. 1), *M. irregularis* sequences grouped closely with those of *M. hiemalis* Wehmer, which has morphological characteristics similar to *M. irregularis*. However, the sporangiophores of *M. hiemalis* are slightly sympodially branched, whereas *M. irregularis* produces monopodially and sympodially branched sporangiophores. The spherical to globose, grayish brown sporangia with diffluent walls of *M. irregularis* contrast to the globose, yellowish to dark brown sporangia with deliquescent walls observed in *M. hiemalis*. *Mucor irregularis* produces predominantly globose and ellipsoidal to cylindrical columellae that differ from the ellipsoidal with the truncate base type found in *M. hiemalis*. Additionally, *M. hiemalis* produces ellipsoidal sporangiospores (some flattened on one side) with few granules, whereas in *M. irregularis* the sporangiospores are smooth-walled, mostly ellipsoidal, cylindrical or globose (Schipper 1973).

Our Brazilian specimens, isolated from soil and as an endophyte in healthy roots, represent the first South American record of *M. irregularis*.

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